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Analysis of Distinct Tartrate-resistant Acid Phosphatase Promoter Regions in Transgenic Mice*

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The tartrate-resistant acid phosphatase (TRAP) is present in multiple tissues, including kidney, liver, lung, spleen, and bone. Recent study of (TRAP) gene expression has provided evidence for distinct promoters within the (TRAP) gene, suggesting that the gene has alternative, tissue-preferred mRNA transcripts. Examination of endogenous (TRAP) exon 1B and 1C mRNA transcripts revealed tissue-preferred transcript abundance with increased exon 1B transcripts detected in liver and kidney and increased exon 1C transcripts detected in bone and spleen. In this investigation, we have made transgenic mice that express a marker gene driven by two candidate promoters, designated BC and C, within the (TRAP) gene. The BC and C promoters are 2.2 and 1.6 kb, respectively, measured from the translation initiation site. Evaluation of BC transgenic lines demonstrated robust expression in multiple tissues. In contrast, significant transgene expression was not detected in C transgenic lines. Evaluation of transgene mRNAs in BC transgenic lines revealed that virtually all expression was in the form of B transcripts, suggesting that the tissue-preferred pattern of endogenous (TRAP) was not replicated in the BC transgenic line. Likewise, osteoclastogenic cultures from BC, but not C, transgenic bone marrow cells expressed the transgene following receptor activator of NFκB ligand/macrophage colony-stimulating factor stimulation. In conclusion, when compared with the 2.2-kb BC portion of the (TRAP) promoter region, the 1.6-kb C portion does not account for significant gene expression *in vivo* or *in vitro*; production of the bone- and spleen-preferred (TRAP) C transcript must depend on regulatory elements outside of the 2.2-kb promoter. As the majority of currently investigated transcription factors that influence transcriptional regulation of osteoclast gene expression bind within the 1.6-kb C portion of the (TRAP) promoter, it is likely that transcription binding sites outside of the 2.2-kb region will have profound effects on regulation of the gene *in vivo* and *in vitro*.

Tartrate-resistant acid phosphatase (TRAP)[1] is highly expressed in multiple tissues, including kidney, liver, lung, pancreas, spleen, bone, and intestine (1, 2). TRAP activity in the liver and kidney is predominantly in liver parenchymal cells and glomerular mesangial cells, respectively. TRAP activity in spleen, lung, and pancreas has been credited to mononuclear phagocytes (2), and TRAP expression in bone is attributed to osteoclasts and their precursor cells.

Several mammalian TRAP genes have been cloned, and the intron-exon structure of the TRAP gene is highly conserved in all species studied to date, including mouse, rat, pig, and human (3–7). Endogenous TRAP expression, based on detection of TRAP mRNA, has been revealed in a variety of tissues, including adult and neonatal bone, heart, brain, kidney, liver, lung, and spleen. Among these tissues, TRAP mRNA is most abundant in the liver, kidney, and bone (2, 8). Investigations using transgenic mice have employed 2-kb TRAP promoters to express selected genes in a tissue-restricted fashion. Sites of transgene expression have included bone, liver, pancreas, spleen, lung, heart, brain, colon, brown fat, kidney, small intestine, and thymus (2, 9, 10).

Recent study of murine TRAP gene expression suggests that there may be tissue-specific TRAP promoters (8); as a result, it has been proposed that the TRAP gene has three alternative 5′-untranslated regions that align with the first base of exon 2 (Fig. 1). These have been designated exons 1A, 1B, and 1C (8). It has been proposed that the promoter for exon 1B is responsible for basal level TRAP mRNA expression in macrophages and osteoclasts and is the major promoter utilized in nonhematopoietic cells. The exon 1C promoter is suspected to contribute to regulation of TRAP expression beyond basal expression in hematopoietic cells, such as macrophages and osteoclasts (8). The exon 1A promoter function is unknown, as 1A mRNA transcripts were detected in adult mouse bone but not *in vitro* or in neonatal bone.

To improve understanding of transcriptional regulation of TRAP gene expression, we generated transgenic mice with distinct TRAP promoter regions fused to a reporter gene. Promoter regions consisted of the 2.2- or the 1.6-kb (relative to ATG) regions of the TRAP promoter region. The 2.2-kb region contained exons 1B and 1C, and the 1.6-kb region contained only exon 1C (Fig. 1). Transgenic lines derived from animals with the 2.2-kb promoter (BC mice) demonstrated robust tissue-restricted reporter gene expression, whereas transgenic

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[1] The abbreviations used are: TRAP, tartrate-resistant acid phosphatase; NGFR, human nerve growth factor receptor; CDy, yeast cytosine deaminase; RANKL, receptor activator of NFκB ligand; MCSF, macrophage colony-stimulating factor; 5FC, 5-fluorocytosine; TG, transgenic; HPRT, hypoxanthine-guanine phosphoribosyltransferase.

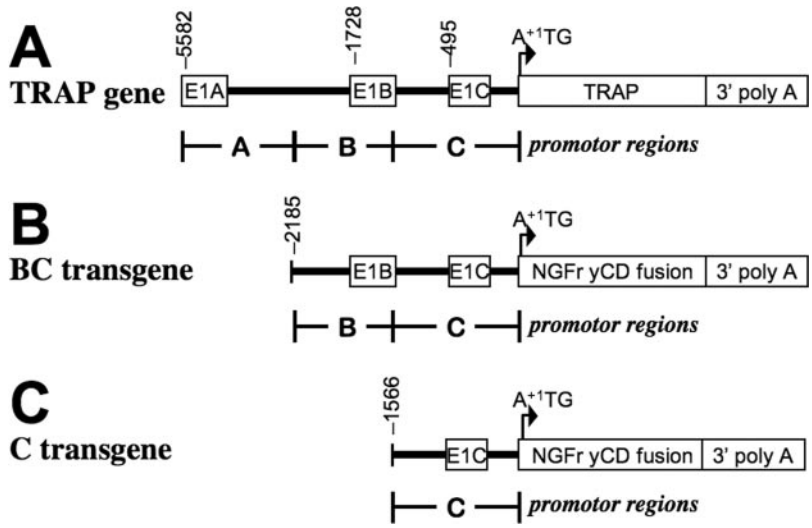

FIG. 1. **Schematic diagram of the mouse TRAP gene and transgene constructs.** *A*, overview of the mouse TRAP gene promoter (8), which consists of three exons, and the translation initiation site ($A^{+1}TG$) located at the beginning of exon 2. The three TRAP mRNA transcripts include exons 1A, 1B, or 1C. *B*, transgene BC consists of TRAP gene 1B and 1C promoter regions, an NGFR·CDy fusion gene replacing the coding region for TRAP, and the 3′-poly(A) tail. *C*, transgene C is similar to BC except the 1B promoter region has been deleted.

lines derived from animals with the 1.6-kb promoter (C mice) failed to express significant gene expression. These findings provided novel insight into understanding transcriptional regulation of the TRAP gene by establishing that the 2.2-kb promoter is essential for gene expression *in vivo*, by determining that the 1.6-kb promoter region is not sufficient for gene expression *in vivo*, and by suggesting that expression of the bone- and spleen-preferred TRAP C transcript must depend on regulatory elements outside of the 2.2-kb TRAP promoter.

MATERIALS AND METHODS

Generation and Identification of Transgenic Mice—Two transgene constructs comprised of a portion of the murine TRAP promoter and a fusion gene containing a marker gene and a therapeutic gene were used to generate mice. A plasmid, LNGCDySN, containing the NGFR·CDy fusion gene was provided by Paul Orchard (11). The NGFR·CDy fusion gene contained the truncated human nerve growth factor receptor (NGFR) gene and the yeast (y) gene encoding cytosine deaminase (CDy). The fusion gene was amplified by polymerase chain reaction (PCR), using a sense oligonucleotide, Koz-NGFR-5′-HinDIII (AAGCTTCCACCATGGGGGCAGGTGCCA) and an antisense oligonucleotide, CDy-3′-HinDIII (AAGCTTCGATCACTCACCAATATCTTC) and cloned into pTRAP vector. The construct was designated BC-NGFR·CDy. pTRAP contains one promoter fragment (−1285 to −1 relative to ATG) and one 3′-poly(A) tail fragment (+2866 to +3385 relative to ATG) from mouse TRAP gene in pBluescriptSK. The second transgene, designated C-NGFR·CDy, was generated by deletion of a 620-bp fragment (−2160 to −1541 relative to ATG) from BC-NGFR·CDy. The BC-NGFR·CDy and C-NGFR·CDy transgenes were excised by removing pBluescriptSK, purified with a QiAquick gel extraction kit (catalogue number 28706; Qiagen Inc., Valencia, CA), and purified DNA was diluted to 4 ng/μl in a TE buffer (5 mM Tris, pH 7.5, 0.1 mM EDTA, pH 8.0).

Transgenic mice were produced by pronuclear microinjection of purified DNA into C3H/HeJ(F) × C57B1/6 (M) embryo eggs by the University of Minnesota Mouse Genetics Laboratory. Founder mice and offspring were identified by Southern blot.

Real-time Quantitative RT-PCR—RNA was isolated and purified using TRIzol (Invitrogen). Expression levels of mRNA were determined using two-step qPCR, with SYBR green as a detection method. cDNA templates for PCR amplification were prepared using a Retroscript™ first strand synthesis kit for RT-PCR (Ambion Inc., Austin, TX). Random decamers were used to amplify 1.25 μg of total RNA according to the manufacturer's instructions in a reaction volume of 20 μl. 2 μl of this cDNA reaction volume were added to 23 μl of a PCR master mix for a final real-time quantitative reaction volume of 25 μl. The PCR master mix was prepared using Qiagen's Quantitect™ SYBR® green PCR kit (Qiagen). Four sets of primer pairs were used: NGFR·CDy-F3, CAGAACAAGACCTCATAGCC and NGFR·CDy-R1, GACATCCGCCAATAGGAACA (NGFR·CDy fusion gene); TRAP 1B-F, GCGCCGCCTCTTCCCAACTC and NGFR·CDy-R4, CGGAGAACGTCACGCTGTCCA (exon 1B transgene transcript); TRAP 1C-F, ACTGCCTCTTGCAGCCTCTC and NGFR·CDy-R3, ACTCGGTGCACGGCTTGCAC (exon 1C transgene transcript); and HPRT-F, GTAATGATCAGTCAACGGGGGAC and HPRT-R, CCAGCAAGCTTGCAACCTTAACCA (HPRT). HPRT was used for comparison and determination of relative ratio between samples. All primers used had a final concentration of 0.3 μM. Real-time quantitative RT-PCR analysis was performed using the ABI PRISM 7900 sequence detection system instrument and software (PE Applied Biosystems, Inc., Foster City, CA). Thermal cycling conditions were set according to the manufacturer's directions. Quantitated mRNA values were normalized by the amounts of HPRT mRNA, and results are given as -fold induction.

Western Analysis—Transgenic mouse tissue (at least 100 mg) was homogenized in 0.5 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, 1 mM NaF, 1 mM Na_3PO_4) containing protease inhibitor mixture tablets (Roche Applied Science). The homogenate was allowed to settle on ice for 20 min and then was centrifuged for 2 min at 14,000 × *g* in a microcentrifuge at 4 °C. Supernatant was decanted and stored at −80 °C. 700 μg of cell lysate/lane was separated on a 10% reducing SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). Polyclonal anti-cytosine deaminase (catalogue number 2485-4906; BIOTREND, Destin, FL) were applied at dilutions of 1:1000 and bovine anti-sheep-horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:3000.

Cell Culture—To determine whether transgenic (TG) bone marrow expressed NGFR *in vitro*, replicate wells containing 4.5 × 10^4 TG or C3H/HeJ bone marrow cells, 20 ng/ml receptor activator of NFκB ligand (RANKL) (Amgen Corp., Thousand Oaks, CA), and 30 ng/ml macrophage colony-stimulating factor (MCSF) (R&D Systems, Minneapolis, MN) in 0.2 ml were cultured in 96-well plates. After 6 days, wells were stained for TRAP (Sigma) or NGFR immunocytochemistry.

NGFR Immunocytochemistry—Monoclonal mouse anti-human NGFR (clone 20.4, provided by Dr. Paul Orchard) was diluted 1:500 and incubated at 37 °C for 1 h. A biotinylated goat anti-mouse IgG (Rockland) was used as the secondary antibody (1:750 dilution, 30 min at 37 °C), and the tertiary reagent was horseradish peroxidase avidin-biotin complex (HRP-ABC; Vector Labs, Burlingame, CA).

In Vitro Cytotoxicity Assay—To assess the effect of 5-fluorocytosine (5FC) on osteoclastogenesis, TG or C3H/HeJ bone marrow cells (4.5 × 10^4/well), RANKL, and MCSF were cultured in 96-well plates with or without various doses of 5FC (0.05–1.0 mM). TRAP solution assay was performed on day 6 and enzyme activity read using an enzyme-linked immunosorbent assay plate reader at 405 nm (12). Cytotoxicity was determined by calculation of percent osteoclasts, as measured against wells containing no drug.

Immunohistochemistry—Paraffin-embedded sections were cut at 6 μm and mounted onto SuperfrostPlus™ slides as described previously (13).

Statistics—Replicate wells were averaged for each drug dose, and data are presented as mean ± S.E. of three or four different animals. Statistical significance was determined by Student's *t* test. A *p* value <0.05 was considered statistically significant.

RESULTS

Generation of Transgenic Lines—medTransgenic lines containing BC-NGFR·CDy or C-NGFR·CDy were generated by microinjection of either construct into fertilized C57BL/6 × C3H/

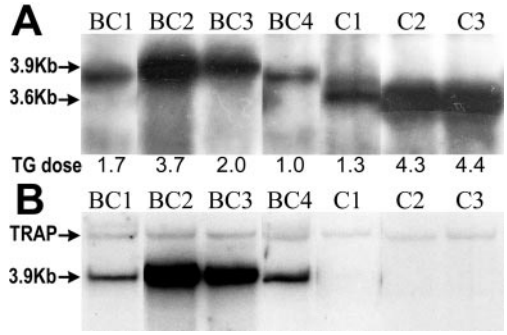

FIG. 2. **Southern blot analysis of BC and C transgenes in transgenic lines.** *A*, analysis of four BC and three C lines using a probe specific for the NGFR·CDy fusion gene. This probe results in a 3.9-kb band in BC mice and a 3.5-kb band in C lines. The relative transgene dosage (*TG dose*) was estimated by comparing band intensity, with BC4 referenced as 1. *B*, analysis of mouse lines using a probe specific for the 1B region of the TRAP promoter. As expected, only BC lines show the 3.9-kb band of the transgene, and all transgenic lines show a faint band representing endogenous TRAP gene (*TRAP*).

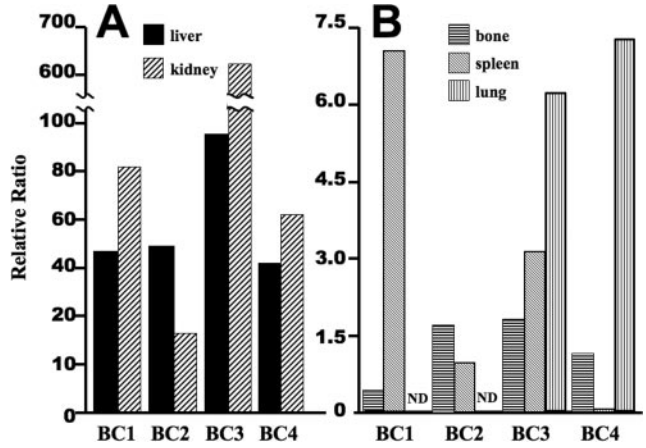

FIG. 3. **Comparison of total mRNA expression for the transgene in BC transgenic lines.** mRNA expression using primer pairs for the NGFR·CDy fusion gene shows high expression in liver and kidney (*A*) and lower (note scales) expression in bone, spleen, and lung (*B*) in all four BC lines. Ratios are relative to HPRT.

HeJ F_1 mouse embryos. The presence of the transgene was determined by Southern blot. Founder mice were mated with C3H/HeJ mice, and the transgene in offspring was determined by PCR. The four BC-NGFR·CDy mouse lines containing the B and C regions of the TRAP promoters were designated BC1–4, and the three C-NGFR·CDy mouse lines containing the C region of the TRAP promoter were designated C1–3.

Southern blot analysis probing for the NGFR portion of each transgene demonstrated that the relative gene dosages in the BC-NGFR·CDy and C-NGFR·CDy transgenic lines spanned similar ranges. The estimated relative gene dosages for mice with the 2.2-kb promoter (BC) ranged from 1.0 to 3.7, and the estimated relative gene dosage for mice with the 1.6-kb promoter (C) ranged from 1.3 to 4.4 (Fig. 2). Southern probe targeting the B portion of the TRAP promoter detected endogenous TRAP gene in all lines, no gene in the C lines (as expected), and transgene in each of the BC lines (Fig. 2). The relative gene dose for BC lines based on Southern blots probed for the B region was qualitatively similar to gene dose estimates based on Southern blots probed for detection of NGFR.

Comparison of Transgene Expression in BC and C Transgenic Mice—Transgene expression was higher in BC mice than in C mice. mRNA expression in kidney, liver, lung, spleen, bone, brain, and heart was determined in seven lines of mice. Quantitative real-time RT-PCR using primers for total NGFR·CDy mRNA revealed easily quantifiable expression of the transgene in each tissue examined from BC transgenic mice. Highest transgene expression was noted in liver and kidney (Fig. 3). Significant expression was seen in the lung, spleen, and bone. Minimal expression was present in the heart and brain. In contrast, transgene expression in C transgenic mice was not detectable in liver, kidney, spleen, lung, bone, and heart and was minimally detectable in brain. Evaluation of endogenous TRAP mRNA transcript expression in the transgenic mice revealed expected findings and confirmed a previous report describing transcript distribution (8).

Total Transgene Transcript Expression in BC Mice—A general pattern of gene expression was noted in each of the four BC transgenic lines. Highest expression was detected in kidney and liver; lower expression was noted in lung, bone, and spleen (Fig. 3). Specifically, in three of the four BC lines, transgene expression was greatest in the kidney. In one line, expression was greatest in the liver. In all four BC lines, expression in the liver and kidney ranged from 10 to several hundred times greater than expression in lung, spleen, or bone.

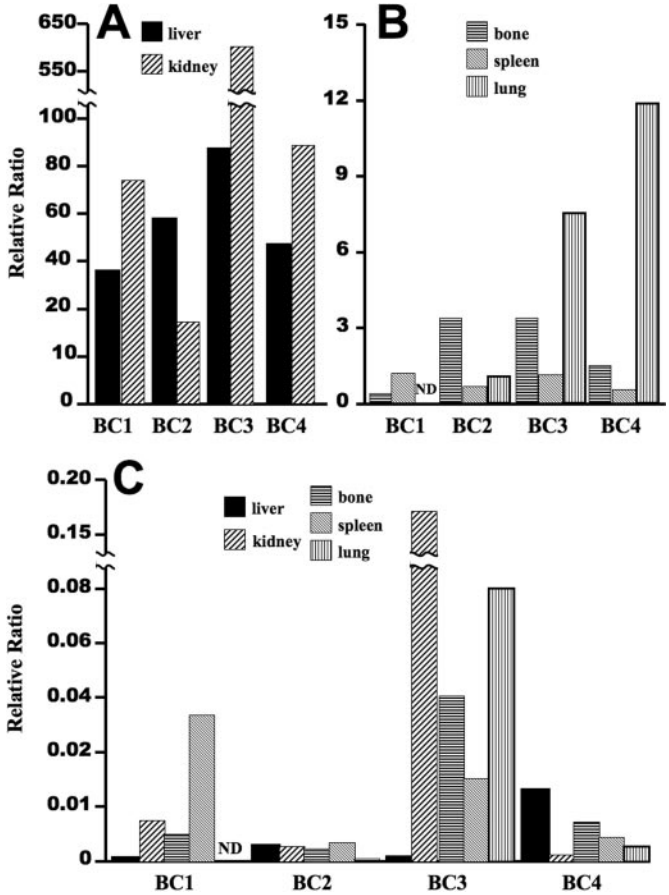

FIG. 4. **Comparison of exon 1B and 1C transgene transcripts in BC transgenic lines.** Quantitation of mRNA using primers specific for exon 1B transgene transcript (*A* and *B*) or exon 1C transgene transcript (*C*) in different tissues. Note differences in relative ratio scales. Ratios are relative to HPRT.

Exon 1B and Exon 1C Transcript Expression in BC Mice—To determine relative transcript expression in the BC transgenic mice, quantitative real time PCR for the exon 1B or 1C transcripts was performed. Findings indicate that the exon 1B transcripts were the dominant transcript in each BC line. Measurements of exon 1B transcript expression in each of the four BC lines provided data mirroring findings from detection of the NGFR transcript (Fig. 4, *A* and *B*). Between mice in each

BC line, kidney and liver had the highest levels of expression. Expression levels ranged from 10- to several hundred-fold higher than spleen, lung, or bone in each animal. Evaluation of exon 1C transcript levels revealed minimal expression in all tissues and did not show evidence of tissue-specific transcript expression (Fig. 4*C*).

Detection of NGFR·CDy Fusion Protein in Transgenic Mice—Western blot analysis of CDy protein demonstrated protein in the liver, kidney, and bone of BC mice (Fig. 5*A*). Western blot analysis did not detect protein in any tissue from C mice (data not shown). The NGFR·CDy fusion protein is commonly detected as two bands (13). Interestingly, kidney and bone displayed a single 63-kDa band, compared with liver which displayed 67 and 63-kDa bands, suggesting that there is tissue-specific post-translational modification of the NGFR·CDy fusion protein. Immunohistochemical detection of NGFR localized protein to the cell membrane of hepatocytes and renal tubular cells (Fig. 5, *B–E*) in BC, but not in C, mice.

Evaluation of Transgene Expression in Vitro—Under osteoclastogenic conditions *in vitro*, cells from BC and C transgenics had increased expression of the endogenous TRAP gene, but only cells from BC mice exhibited increased expression of the NGFR·CD transgene. To induce osteoclast or macrophage differentiation, bone marrow cells from BC or C mice were cultured in the presence of MCSF and RANKL or MCSF alone, respectively. Endogenous TRAP expression increased in osteoclastogenic, but not macrophage, cultures containing cells from BC or C mice (Fig. 6*A*). In culture systems from BC mice, transgene expression increased significantly upon exposure to MCSF alone (Fig. 6*B*). Interestingly, in culture systems from C mice, transgene induction was not observed under either osteoclastogenic or macrophage differentiation conditions (Fig. 6*B*). To further characterize the effect of RANKL and MCSF treatment on TRAP and transgene expression *in vitro*, detection of the B and C transcripts was performed in normal and BC cells. Findings revealed that the endogenous exon 1B and exon 1C TRAP transcripts were induced following RANKL and MCSF treatment (Fig. 6, *C* and *D*). Likewise, expression of the transgenic exon 1B and 1C transcripts was also induced under osteoclastogenic conditions (Fig. 6, *E* and *F*).

Detection of Transgenic Fusion Protein in Vitro—Findings from histochemical and immunohistochemical evaluation of osteoclastogenic culture systems were consistent with data from *evaluation of gene expression. TRAP enzyme detection demonstrated that osteoclasts formed in culture (Fig. 7, B* and *D*). Immunohistochemical detection of the NGFR portion of the NGFR·CDy fusion protein demonstrated the presence of the fusion protein in osteoclastogenic culture systems derived from BC mice but, as expected, not from C3H control mice (Fig. 7, *A* and *C*).

Detection of the CDy portion of the NGFR·CDy fusion protein was performed using an assay based on cytosine deaminase enzyme activity. As CDy converts metabolically inert 5-fluorocytosine to the cytotoxic agent 5-fluorouracil, exposure of CDy-expressing osteoclastogenic cell culture systems to 5FC should result in a reduction in osteoclast number (13). Treatment of osteoclastogenic culture systems from nontransgenic mice had no effect on osteoclast number. In contrast, treatment of osteoclastogenic culture systems derived from BC mice with 5FC resulted in reduction in the number of osteoclasts (Fig. 7*E*). 5FC doses as low as 0.05 mM eliminated 60% of the osteoclasts in BC culture systems, and the maximal effect was seen with doses at or above 0.1 mM 5FC.

DISCUSSION

The primary objective of this investigation was to compare transgene expression in transgenic mice with a reporter gene

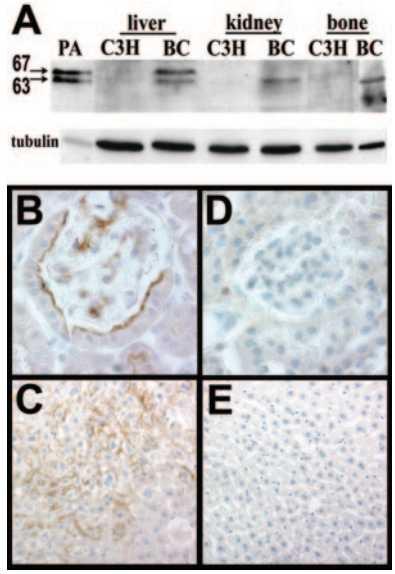

FIG. 5. **Demonstration of NGFR·CDy fusion protein in BC line.** *A*, Western analysis for CDy shows expression in liver, kidney, lung, and bone from BC (*lanes 3*, *5*, *7*) mice, but not control C3H. NGFR·CDy-expressing cell line is shown as a positive control for Cdy, and α-tubulin is used as the loading control. Immunohistochemical staining (*brown*) for NGFR in kidney (*B*, *D*) and liver (*C*, *E*) from BC (*B*, *C*) or C mouse (*D*, *E*). No staining is present in C mice. Kidney ×1000 and liver ×400 magnification.

(NGFR·CDy) regulated by either the 2.2- (BC) or the 1.6-kb (C) portion of the TRAP promoter. Findings demonstrated that transgene expression was robust in mice with the BC promoter and that transgene expression was essentially absent in mice with the C promoter. Immunohistochemistry of liver and kidney detected NGFR in BC, but not C, mice. *In vitro* osteoclastogenic conditions stimulated significant transgene expression in marrow cells from BC, but not C, mice and immunohistochemical detection of NGFR-positive osteoclasts.

Our analysis of gene expression driven by the 2.2- and 1.6-kb regions of the TRAP promoter indicated robust expression from the BC promoter and nil expression from the C promoter *in vivo*. This finding was unexpected, as a previous analysis of TRAP mRNA from various mouse tissues provided evidence that the C region of the TRAP promoter may be sufficient for substantial gene expression (8). Low expression of the transgene in our C mice could not be explained by relative transgene copy dose, as estimates of transgene copy dosage range were similar in the BC and C mice. The dominant transgenic transcript in BC mice was the B transcript, with C transcripts being barely detectable. This finding was particularly interesting because analysis of endogenous TRAP mRNA transcripts in the transgenic mice indicated that endogenous C transcripts were abundant in bone and spleen, as has previously been reported (8).

Based on previous determination of the relative abundance of distinct TRAP mRNA transcripts and our current analysis of endogenous TRAP transcript expression, we expected to observe that tissue- or cell type-specific transcripts would be present in hemopoietic (bone, spleen) *versus* nonhematopoietic (kidney, liver) tissues (8). In contrast, we determined that transcripts derived from the 2.2-kb promoter region showed near complete fidelity to exon 1B. Taken in total, our *in vivo* finding of limited exon 1C transcript levels suggested that the most 5′-portion of the TRAP gene containing −6.5 to −2.2-kb (relative to ATG) may be required for generation of the exon 1C transcript.

Several transcription factors involved in osteoclast formation

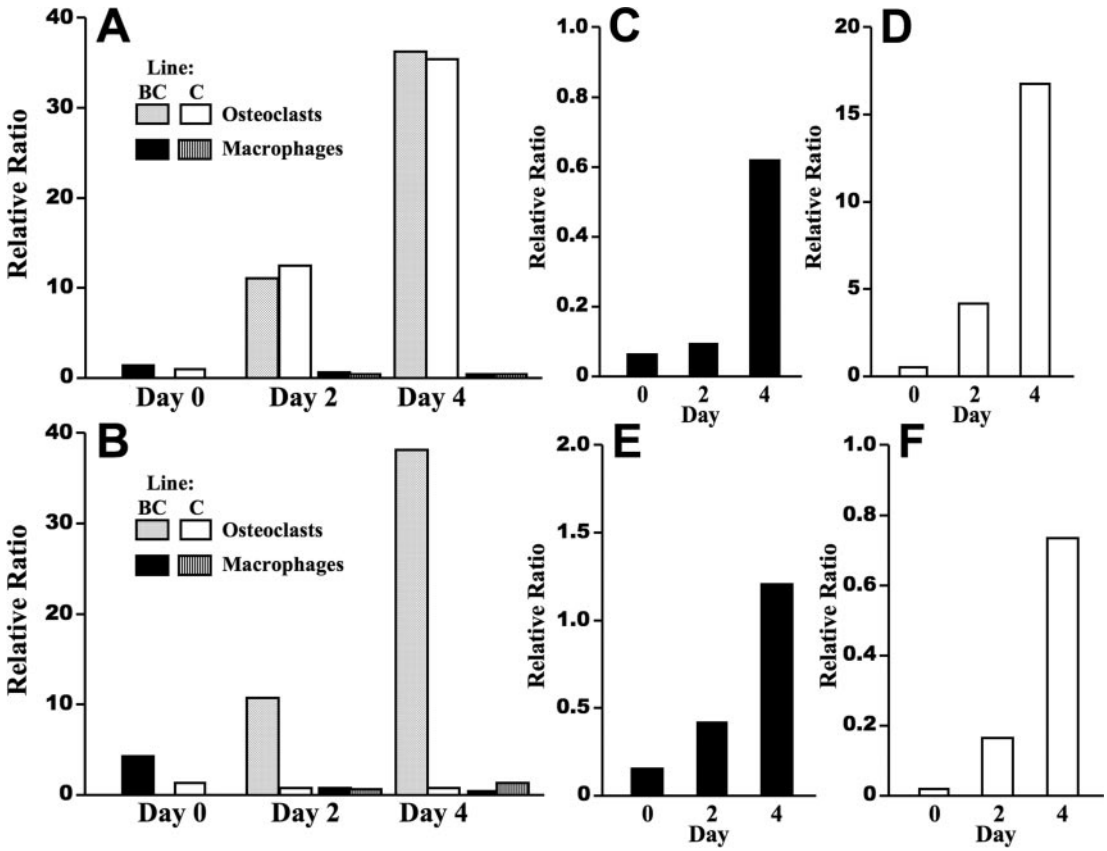

FIG. 6. **Transgene expression in osteoclastogenic culture from BC and C mice.** Levels of mRNA for total endogenous TRAP (*A*) and total NGFR·CDy (*B*) is shown in osteoclasts and macrophages of BC and C mice. Endogenous TRAP B transcript (*C*), endogenous TRAP C transcript (*D*), NGFR·CDy B transcript (*E*), and NGFR·CDy C transcript (*F*) is shown in osteoclasts of BC mice. Expression ratios are relative to HPRT.

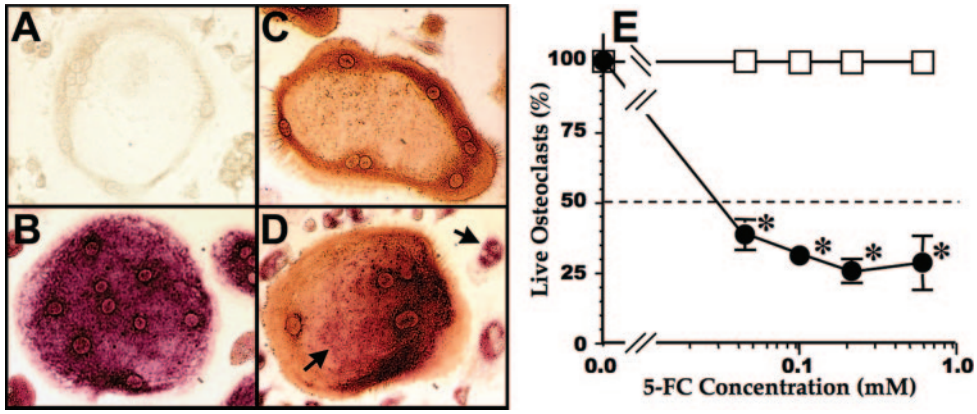

FIG. 7. **Detection of NGFR·CDy fusion protein *in vitro*.** *A*, bone marrow from control (C3H/HeJ) and BC transgenic mouse bone marrow was grown *in vitro* for 6 days with RANKL and MCSF. Replicate wells of osteoclasts from C3H/HeJ (*A*, *B*) and BC (*C*, *D*) mice were stained for NGFR (*A*, *C*), TRAP (*B*), or NGFR and TRAP (*D*). Osteoclasts (*garnet-stained* multinucleated cells) formed in all wells. However, NGFR (*brown*) is present only in osteoclasts formed from transgenic bone marrow (×400). Double-positive staining for TRAP (*arrows*) and NGFR can be seen in osteoclasts from BC mice (*D*). *E*, activity of CDy was assessed in osteoclastogenic cultures. Bone marrow from C3H/HeJ (-□-), or BC (-●-) mice was cultured in the presence of RANKL (20 ng/ml) and MCSF (30 ng/ml) with increasing doses of 5FC. *, $p < 0.01$, $n = 4$.

and activity have been shown to interact with the TRAP promoter. These include MITF, Pip, PU.1, USF1, USF2, TFE3, and TFEC (14–18). Each of these DNA binding proteins has been studied in *in vitro* TRAP promoter-reporter experimental systems, and transcription factor binding sites have been localized to the 3′-end of the TRAP promoter within the C region. As our findings demonstrated that the C region of the TRAP promoter alone is not sufficient for transgene expression, further investigation is warranted to decipher the transcription factor binding sites within the B region that permit vigorous TRAP gene expression *in vivo*.

It is becoming increasingly clear that a complex set of regulatory mechanisms controls TRAP gene expression and transcriptional events in TRAP-expressing cells (8). This work has improved our understanding of TRAP gene regulation in osteoclasts by demonstrating that the 1.6-kb promoter region is not sufficient for expression of the TRAP gene *in vivo* and *in vitro* and by showing that the exon 1B transcript is the predominant TRAP mRNA in transgenic mice with the 2.2-kb TRAP promoter. As the great majority of currently studied transcription factors that influence TRAP gene expression bind within the 1.6-kb C region of the TRAP promoter, our findings strongly

suggested that transcription factor binding sites upstream of the C region will also have profound effects on regulation of the TRAP gene.

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